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(54) Title: IMMUNOTHERAPY INVOLVING CD28 STIMULATION

(57) Abstract

A method of immunotherapy stimulates the T cell CD28 surface molecule to enhance T cell proliferation and increase overall lymphokine levels or to increase cellular production of human T_H1 lymphokines or both. The method is selective for the induction of activated T cell mediated immune responses and enhances immune function even in the presence of immunosuppressants.

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IMMUNOTHERAPY INVOLVING CD28 STIMULATION

Background of the Invention

The present invention generally relates to immunotherapy. More particularly, the present invention relates to a method of immunotherapy involving stimulation of the CD28 T cell surface molecule to augment the T cell-mediated immune response in vivo.

Thymus derived lymphocytes, referred to as T cells, are important regulators of in vivo immune responses. T cells are involved in cytotoxicity and delayed type hypersensitivity (DTH), and provide helper functions for B lymphocyte antibody production. In addition, T cells produce a variety of lymphokines which function as immunomodulatory molecules, such as for example, interleukin-2 (IL-2), which can facilitate the cell cycle progression of T cells; tumor necrosis factor-alpha (TNF-α) and lymphotoxin (LT), cytokines shown to be involved in the lysis of tumor cells; interferon-gamma (IFN-γ), which displays a wide variety of anti-viral and anti-tumor effects; and granulocyte-macrophage colony stimulating factor (GM-CSF), which functions as a multilineage hematopoietic factor.

Current immunotherapeutic treatments for diseases such as cancer, acquired immunodeficiency syndrome (AIDS) and attending infections, involve the systemic administration of lymphokines, such as IL-2 and IFN-γ, in an attempt to enhance the immune response by T cell proliferation. However, such treatment results in non-specific augmentation of the T cell-mediated immune response, since the lymphokines administered are not specifically directed against activated T cells proximate to the site of infection or the tumor. In addition, systemic infusions of these molecules in pharmacologic doses leads to significant toxicity. Present therapies for immunodeficient or immunodepressed patients also involve non-specific augmentation of the immune system using concentrated gamma globulin preparations or the systemic infusion of T cell lymphokines with disadvantageous systemic side effects. The stimulation of the in vivo secretion of immunomodulatory factors has not, until now, been considered a feasible alternative due to the failure to appreciate the effects and/or mechanism and attending benefits of such therapy.

It would thus be desirable to provide a method of immunotherapy which enhances the T-cell mediated immune response and which is directed specifically toward T-cells activated by an antigen

produced by the targeted cell. It would further be desirable to provide a method of immunotherapy which could take advantage of the patient's natural immunospecificity. It would also be desirable to provide a method of immunotherapy which can be used in immunodepressed patients. It would additionally be desirable to provide a method of immunotherapy which does not primarily rely on the administration of immunomodulatory molecules in amounts having significant toxic effects.

It would also be desirable to provide a method of immunotherapy which, if so desired, could be administered directly without removal and reintroduction of T cell populations. It would further be desirable to provide a method of immunotherapy which could be used not only to enhance, but to suppress T-cell mediated immunoresponses where such immunosuppression would be advantageous, for example, in transplant patients and in patients exhibiting shock syndrome.

Summary of the Invention

The immunotherapeutic method of the present invention comprises the step of selectively regulating the in vivo level of a human T-cell lymphokine by administering a therapeutically effective amount of a ligand to a patient having a population of activated T cells, said ligand having binding specificity for at least a portion of the extracellular domain of the CD28 T-cell surface molecule.

The method of immunotherapy of the present invention takes advantage of the surprising and heretofore unappreciated effects of stimulation of the CD28 molecule of activated T cells. By activated T cells is meant cells in which the immune response has been initiated or "activated", generally by the interaction of the T cell receptor TCR/CD3 T cell surface complex with a foreign antigen or its equivalent. Such activation results in T cell proliferation and the induction of T cell effector functions such as lymphokine production.

Stimulation of the CD28 cell surface molecule with anti-CD28 antibody results in a marked increase of T cell proliferation and in IL-2 lymphokine levels when the T cell is activated by submaximal stimulation of its TCR/CD3 complex. Surprisingly, when the stimulation of the TCR/CD3 complex is maximized, upon co-

stimulation with anti-CD28 there is a substantial increase in the levels of IL-2 lymphokine, although there is no significant increase in T cell proliferation over that induced by anti-CD3 alone. Even more surprisingly, not only are IL-2 levels significantly increased, but the levels of an entire set of lymphokines previously not associated with CD28 stimulation are increased. Remarkably both the T cell proliferation and increased lymphokine production attributable to CD28 stimulation also exhibit resistance to immunosuppression by cyclosporine and glucocorticoids.

The method of immunotherapy of the present invention thus provides a method by which the T cell-mediated immune response can be regulated by stimulating the CD28 T cell surface molecule to aid the body in ridding itself of infection or cancer. The method of the present invention can also be used not only to increase T cell proliferation, if so desired, but to augment the immune response by increasing the levels and production of an entire set of T cell lymphokines now known to be regulated by CD28 stimulation.

Moreover, because the effectiveness of CD28 stimulation in enhancing the T cell immune response appears to require T cell activation or some form of stimulation of the TCR/CD3 complex, the method of immunotherapy of the present invention can be used to selectively stimulate preactivated T cells capable of protecting the body against a particular infection or cancer, thereby avoiding the non-specific toxicities of the methods presently used to augment immune function. In addition, the method of immunotherapy of the present invention enhances T cell-mediated immune functions even under immunosuppressed conditions, thus being of particular benefit to individuals suffering from immunodeficiencies such as AIDS.

A better understanding of the present invention and its advantages will be had from a reading of the detailed description of the preferred embodiments taken in conjunction with the drawings and specific example set forth below.

Brief Description of the Drawings

Figure 1 is a bar graph illustrating the absence of augmentation of the uptake of thymidine by CD28 stimulated T cells.

Figure 2 is a bar graph illustrating the increase in uridine incorporation by CD28 stimulation of anti-CD3 stimulated T

cells.

Figure 3 is a graph illustrating the elevated cyclosporine resistance of T cell proliferation induced by CD28 stimulation.

Figure 4 is a Northern blot illustrating the effects of cyclosporine on PMA or anti-CD3 activated T cells lymphokine expression induced by anti-CD28.

Figure 5 is a graph of in vivo activation of T cells in monkeys by CD28 stimulation.

Detailed Description of the Preferred Embodiments

In a preferred embodiment of the immunotherapeutic method of the present invention, the CD28 molecule is stimulated to enhance the T cell-mediated immune response of antigen-activated T cells or their equivalent. CD28 is a 44 kilodalton protein expressed on the surface of about 80% mature T cells which exhibits substantial homology to immunogloblin genes. See Poggi, A., et al., Eur. J. Immunol., 17:1065-1068 (1987) and Aruffo, A., et al., PNAS (USA), 8573-8577 (1987), both herein incorporated by reference. Binding of the CD28 molecule's extracellular domain with anti-CD28 antibodies in accordance with the method of the present invention results in an increase in T cell proliferation and elevated lymphokine levels.

In Specific Examples III-IV and VI-VIII, T cell activation was accomplished by stimulating the T cell TCR/CD3 complex (which mediates the specificity of the T cell immune response) with immobilized anti-CD3 monoclonal antibodies, such as mAb G19-4, or by chemically stimulating with PMA and ionomycin. It should also be appreciated, however, that activation of the T cell can instead be accomplished by routes that do not directly involve CD3 stimulation, such as the stimulation of the CD2 surface protein.

In practice, however, an activated T cell population will be provided by the patient's own immune system, which, barring total immunosuppression, will have T cells activated in response to any foreign or substantially elevated level of antigen present due to disease or infection. The term "foreign antigen" is used broadly herein, meaning an antigen which is either not normally produced by the organism, or, as in carcinomas, an antigen which is not normally produced by the cell which is producing it. By "substantially elevated" level of antigen is meant an antigen level exceeding normal

ranges and having potentially deleterious effects to the organism due to such elevation.

In accordance with the method of the present invention, stimulation of the CD28 molecule itself is achieved by administration of a ligand, such as a monoclonal antibody or a portion thereof, having a binding specificity for CD28. Suitable antibodies include mAb 9.3, an IgG2a antibody which has been widely distributed and is available (for non-commercial purposes) upon request from Dr. Jeffrey A. Ledbetter of Oncogen Corporation, Seattle, WA, or mAb KOLT-2. Both these monoclonal antibodies have been shown to have binding specificity for the extracellular domain of CD28 as described in Leukocyte Typing II, Ch. 12, pgs. 147-156, ed. Reinhertz, E. L., et al. (1986). The $F(ab')_2$ fragment of mAb 9.3 is at present preferred, having been tested in vivo without adverse side effects reported. It should also be understood that the method of the present invention contemplates the use of chimaeric antibodies as well as non-immunoglobulin ligands which bind the CD28 surface molecule.

The extracellular domain of CD28, which was sequenced by Aruffo, A., et al., PNAS (USA), 84:8573-8577 (1987), generally comprises the following amino acid sequence:

MetLeuArgLeuLeuLeuAlaLeuAsnLeuPheProSerIleGln
ValThrGlyAsnLysIleLeuValLysGlnSerProMetLeuVal
AlaTyrAspAsnAlaValAsnLeuSerCysLysTyrSerTyrAsn
LeuPheSerArgGluPheArgAlaSerLeuHisLysGlyLeuAsp
SerAlaValGluValCysValValTyrGlyAsnTyrSerGlnGln
LeuGlnValTyrSerLysThrGlyPheAsnCysAspGlyLysLeu
GlyAsnGluSerValThrPheTyrLeuGlnAsnLeuTyrValAsn
GlnThrAspIleTyrPheCysLysIleGluValMetTyrProPro
ProTyrLeuAspAsnGluLysSerAsnGlyThrIleIleHisVal
LysGlyLysHisLeuCysProSerProLeuPheProGlyProSer
LysPro

By the term "extracellular domain" as used hereinafter in the specification and claims, is meant the amino acid sequence set forth above, any substantial portion thereof, or any sequence having substantial homology thereto.

As shown by the data of Specific Examples III-V, substantial augmentation of the T cell-mediated immunoresponse by

CD28 stimulation appears specific for activated T cells. Such specificity is of particular clinical importance and is one of the significant advantages of the method of immunotherapy of the present invention. Administration of anti-CD28 antibodies such as mAb 9.3 will specifically augment the response of T cells which are already activated and engaged in the immune response or those in the process of activation. It should, however, also be appreciated that CD28 stimulation may be effective even where the T cells are activated after the binding of the CD28 specific ligand of the present invention to CD28 receptor. Thus, the T cells at or near the tumor site or site of infection, which are being activated by the antigens produced or present at those sites, will be selectively "boosted" by the CD28 stimulation.

As previously discussed and further illustrated by the Specific Examples, the synergistic effect of CD28 stimulation on activated T cells results in increased T cell proliferation and increased IL-2 lymphokine levels when the TCR/CD3 complex is not maximally stimulated. However, when TCR/CD3 stimulation is maximized, although T cell proliferation is not markedly increased, the levels of certain lymphokines are substantially increased, indicating an increase in cellular production of these lymphokines. Thus, in patients undergoing natural maximal TCR/CD3 stimulation or its equivalent and T cell activation <u>in vivo</u> due to disease or infection, the administration of anti-CD28 antibody to stimulate CD28 in accordance with the method of the present invention will result in substantially elevated lymphokine production.

The increase in lymphokine production achieved by administration of CD28 stimulator in accordance with the method of the present invention, as particularly shown in Specific Example III, surprisingly results in the increased production of an entire set of lymphokines, indicating that these lymphokines are under some form of CD28 regulation. This set of lymphokines, which includes IL-2, TNF-α, LT, IFN-γ, and GM-CSF, is somewhat analogous to the T_H1 cell lymphokines present in the mouse which were described by Mosmann, T. R., et al., <u>Immunol. Today</u>, 8:223-227 (1987). Such finding is also buttressed by the lack of increase in human IL-4 production (data not shown) by CD28 stimulation, a lymphokine which is also not produced by the T_H1 cells of the mouse. Thus, for ease of reference, the group

of human lymphokines affected by CD28 stimulation will hereinafter be referred to as human T_H1 lymphokines. It should be appreciated, however, that the term "human T_H1 lymphokines" is not limited to the lymphokines listed above, but is meant to include all human lymphokines whose production is affected or regulated by the binding or stimulation of the CD28 T cell surface molecule. Thus, by administration of anti-CD28 antibodies in accordance with the method of immunotherapy of the present invention, the production and levels of an entire set of human lymphokines can be significantly increased.

The method of immunotherapy of the present invention can also be used to facilitate the T cell-mediated immune response in immunodepressed patients, such as those suffering from AIDS. As shown in Specific Examples VI - VIII, T cell proliferation and the increased levels or production of CD28-regulated lymphokines continue to function even in the presence of immunosuppression such as that caused by cyclosporine or dexamethasone. Thus administration of CD28 stimulators such as mAb 9.3 can be used to treat immunodepressed patients to increase their in vivo lymphokine levels.

In addition, a variety of syndromes including septic shock and tumor-induced cachexia may involve activation of the CD28 pathway and augmented production of potentially toxic levels of lymphokines. Thus down-regulation of the CD28 pathway, by, for example, binding CD28 with a $F(ab')_2$ fragment or a naturally occurring ligand for the CD28 molecule, can also provide immunotherapy for those clinical conditions.

It should be appreciated that administration of an anti-CD28 antibody has not heretofore been seriously contemplated as a potential immunotherapeutic method for the substantial increase of lymphokine levels at the sites of activated T cells. For example, the addition of mAb 9.3 has been thought only to somewhat augment T cell proliferation, not to induce substantial increases in human T_H1 lymphokine production.

Although it is not the intent herein to be bound by any particular mechanism by which CD28 binding regulates the T cell-mediated immune response, a model for the mechanism of stimulation has been postulated and supported with experimental data, some of which is shown in Specific Example VIII.

It has previously been shown that a number of lymphokine

genes undergo more rapid degradation in the cytoplasm than mRNAs from constitutitively expressed housekeeping genes, leading to the hypothesis that the instability of these inducible mRNAs has been selected to allow for rapid regulation of gene expression. It is believed that the mechanism of CD28 regulation herein described and claimed is related to the stabilization of rapidly degradable mRNAs for the set of human T_H1 lymphokines set forth above. To date, it appears no other mechanism in any eukararyotic cell system has been described to demonstrate that a cell surface activation pathway can alter gene expression by inducing specific alteration in mRNA degradation.

As shown in Specific Example IV, co-stimulation of CD28 and CD3 caused an increase in mRNA of the human T_H1 lymphokines which was not the result of a generalized increase in a steady state mRNA expression of all T cell activation-associated genes. The increase was disproportionate and thus could not be accounted for by the increase in percentage of proliferating cells in culture. These data, in addition to further studies not detailed herein, demonstrate that activation of the CD28 surface molecule of activated T cells functions to specifically stabilize lymphokine mRNAs. Increased mRNA stability, i.e. slower degradation thereof, results in increased translation of the mRNA, in turn resulting in increased lymphokine production per cell.

Thus, in accordance with the principles of the present invention, ligands such as mAb 9.3 with binding specificity for the CD28 molecule are administered in a biologically compatible form suitable for administration in vivo to stimulate the CD28 pathway. By "stimulation of the CD28 pathway" is meant the stimulation of the CD28 molecule resulting in increased T cell proliferation or production of human T_H1 lymphokines or both. By "biologically compatible form suitable for administration in vivo" is meant a form of the ligand to be administered in which the toxic effects, if any, are outweighed by the therapeutic effects of the ligand. Administration of the CD28 ligand can be any suitable pharmacological form, which includes but is not limited to intravenous injection of the ligand in solution.

It should be understood that, although the models for CD28 regulation of lymphokine production are described with respect to

stimulation and enhancement of lymphokine levels, down-regulation or inhibition of the CD28 pathway may also be achieved in accordance with the principles of the present invention by the selection of the appropriate ligand for CD28 binding.

SPECIFIC EXAMPLE I

Preparation of CD28 Stimulator Monoclonal Antibody 9.3

The monoclonal antibody (mAb) 9.3, an IgG2a monoclonal antibody which binds to the extracellular domain of the CD28 molecule, was produced by a hybrid cell line originally derived by Hansen et al., as described in Immunogenetics, 10:247-260 (1980). Ascites fluid containing high titer monoclonal antibody 9.3 was prepared by intraperitoneal inoculation of 5-10 x 10^6 hybrid cells into a Balb/C x C57BL/6 F_1 mice which had been primed intraperitoneally with 0.5 ml of Pristane (Aldrich Chemical Co., Milwaukee, WI). The monoclonal antibody 9.3 was purified from ascites fluid on a staphylococcal protein-A sepharose column as described by Hardy, R., Handbook of Experimental Immunology, Ch. 13 (1986).

Prior to use in functional assays, purified mAb 9.3 was dialyzed extensively against phosphate buffered saline (KCl 0.2 grams/liter dH_2O; KH_2PO_4 0.2 grams/liter dH_2O; NaCl 8.0 grams/liter dH2O; $Na_2HPO_4 \cdot 7H_2O$ 2.16 grams/liter dH_2O) and then filtered through a 0.22 cubic micron sterile filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). The mAb 9.3 preparation was cleared of aggregates by centrifugation at 100,000 xg for 45 minutes at 20°C. The resulting purified mAb 9.3 was resuspended in phosphate buffered saline to a final concentration of 200 µg/ml as determined by OD280 analysis and stored at 4°C prior to use.

SPECIFIC EXAMPLE II

Isolation of $CD28^+$ T Cells

Buffy coats were obtained by leukopheresis of healthy donors 21 to 31 years of age. Peripheral blood lymphocytes (PBL), approximately 2.5 x 10^9, were isolated from the buffy coat by Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD)

density gradient centrifugation. The $CD28^+$ subset of T cells was then isolated from the PBL by negative selection using immuno-absorption, taking advantage of the reciprocal and non-overlapping distribution of the CD11 and CD28 surface antigens as described by Yamada et al., Eur. J. Immunol., 15:1164-1688 (1985). PBL were suspended at approximately 20 x 10^6/ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 20mM HEPES buffer (pH 7.4) (GIBCO Laboratories, Grand Island, NY), 5mM EDTA (SIGMA Chemical Co., St. Louis, MO) and 5% heat-activated human AB serum (Pel-Freez, Brown Deer, WI). The cells were incubated at 4°C on a rotator with saturating amounts of monoclonal antibodies 60.1 (anti-CD11a) (see Bernstein, I.D., et al., Leukocyte Typing II, Vol. 3, pgs. 1-25, ed. Reinherz, E. L., et al., (1986); 1F5 (anti-CD20) (see Clark, E. A., et al., PNAS(USA), 82:1766-1770 (1985)); FC-2 (anti-CD16) (see June, C. H., et al., J. Clin. Invest., 77: 1224-1232 (1986)); and anti-CD14 for 20 minutes. This mixture of antibodies coated all B cells, monocytes, large granular lymphocytes and CD28 T cells with mouse immunoglobulin. The cells were washed three times with PBS to remove unbound antibody, and then incubated for 1 hour at 4°C with goat anti-mouse immunoglobulin-coated magnetic particles (Dynal, Inc., Fort Lee, NJ) at a ratio of 3 magnetic particles per cell. Antibody-coated cells that were bound to magnetic particles were then removed by magnetic separation as described by Lea, T., et al., Scan. J. Immunol., 22:207-216 (1985). Typically, approximately 700 x 10^6 $CD28^+$ T cells were recovered.

Cell purification was routinely monitored by flow cytometry and histochemistry. Flow cytometry was performed as described by Ledbetter, J. A. et al., Lymphocyte Surface Antigens, p. 119-129 (ed. Heise, E., 1984). Briefly, $CD28^+$ T cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD2 mAb OKT11 (Coulter, Hialeah, FL) and with FITC-conjugated anti-CD28 mAb 9.3 as described by Goding, J. W., Monoclonal Antibodies Principles and Practice, p. 230 (ed. Goding, J. W., 1983). $CD28^+$ T cells were over 99% positive with FITC-conjugated monoclonal antibody OKT11 and over 98% positive FITC-conjugated monoclonal antibody 9.3 when compared to a non-binding, isotype-matched, FITC-labeled control antibody (Coulter, Hialeah, FL). Residual monocytes were quantitated by staining for non-specific esterase using a commercially available kit

obtained from Sigma Chemical Co., St. Louis, MO and were less than 0.1% in all cell populations used in this study. Viability was approximately 98% as measured by trypan blue exclusion as described by Mishell, B.B., et al., Selected Methods Cell. Immunol., pgs. 16-17 (1980).

SPECIFIC EXAMPLE III

Increased Cellular Production of Human T_H1 Lymphokines by CD28 Stimulation by Monoclonal Antibody 9.3

$CD28^+$ T cells were cultured at approximately 1 x 10^5 cells/well in the presence of various combinations of stimulators. The stimulators included phorbol myristate acetate (PMA) (LC Services Corporation, Woburn, MA) at 3 ng/ml conc.; anti-CD28 mAb 9.3 at 100 ng/ml; anti-CD3 mAb G19-4 at 200 ng/ml which was immobilized by adsorbing to the surface of plastic tissue culture plates as previously described by Geppert, et al., J. Immunol., 138:1660-1666 (1987); also Ledbetter, et al, J. Immunol., 135: 2331-2336 (1985); ionomycin (Iono) (Molecular Probes, Calbiochem, CA) at 100 ng/ml. Culture supernatants were harvested at 24 hours and serial dilutions assayed for the human T_H1 lymphokines.

Specifically, IL-2 was assayed using a bioassay as previously described by Gillis et al., Nature, 268:154-156 (1977). One unit (U) was defined as the amount of IL-2 needed to induce half maximal proliferation of 7 x 10^3 CTLL-2 (a human cytotoxic T cell line) cells at 24 hours of culture. In separate experiments the relative levels of IL-2 for each of the culture conditions above were independently confirmed using a commercially available ELISA assay (Genzyme Corp., Boston, MA). TNF-α/LT levels were measured using a semiautomated L929 fibroblast lytic assay as previously described by Kunkel et al., J. Biol. Chem., 263:5380-5384 (1988). Units of TNF-α/LT were defined using an internal standard for TNF-α (Genzyme Corp., Boston MA). The independent presence of both TNF-α and LT was confirmed by the ability of a monoclonal antibody specific for each cytokine to partially inhibit cell lysis mediated by the supernatant from cells co-stimulated with immobilized anti-CD3 mAb G19-4 and anti-CD28 mAb 9.3. IFN-γ was measured by radioimmunoassay using a commercially available kit (Centocor, Malvern, PA). Units for IFN-γ

were determined from a standard curve using ^{125}I-labeled human IFN-γ provided in the test kit. GM-CSF was detected by stimulation of proliferation of the human GM-CSF-dependent cell line AML-193, as described by Lange et al., Blood, 70:192-199 (1987), in the presence of neutralizing monoclonal antibodies to TNF-α and LT. The ^{3}H-thymidine uptake induced by 10 ng/ml of purified GM-CSF (Genetics Institute, Cambridge, MA) was defined as 100 U. Separate aliquots of cells were recovered 48 hours after stimulation and assayed for the percentage of cells in late stages of the cell cycle ($S+G_2+M$) by staining of cells with propidium iodide and analysis by flow cytometry as previously described by Thompson et al., Nature, 314:363-366 (1985).

As shown in Table 1, CD28 stimulation of CD3 stimulated T cells resulted in marked increases in cellular production of IL-2, TNF-α, IFN-γ and GM-CSF, herein referred to as human T_H1 lymphokines.

TABLE 1

Increased Cellular Production of Human T_H1 Lymphokines by CD28 Stimulation

STIMULUS	IL-2 (U/ml)	TNF-α/LT (U/ml)	IFN-γ (U/ml)	GM-CSF (U/ml)	S+G_2+M (%)
Medium	<2	0	0	0	4.6
PMA	<2	0	0	NT	5.5
Anti-CD28	<2	5	0	0	6.5
Anti-CD28+PMA	435	300	24	150	48.9
Anti-CD3[i]	36	50	24	120	39.7
Anti-CD3[i]+Anti-CD28	1200	400	74	1050	44.7
Ionomycin	<2	0	0	NT	6.6
Ionomycin+PMA	200	5	37	NT	43.6
Ionomycin+PMA+Anti-CD28	1640	320	128	NT	43.5

[i] = immobilized
NT = not tested

SPECIFIC EXAMPLE IV

Comparison of CD28 Stimulation to Stimulation of Other T Cell Surface Molecules

$CD28^+$ T cells were cultured at approximately 1 x 10^5 cells/well in RPMI media containing 5% heat-inactivated fetal calf serum (FCS), PHA 10 μg/ml, PMA 3 ng/ml, ionomycin at 100 ng/ml, anti-CD28 mAb 9.3 100 at ng/ml, or mAb 9.4 specific for CD45 at 1 μg/ml or mAb 9.6 specific for CD2 at 1 μg/ml, or immobilized mAb G19-4 specific for CD3 at 200 ng/well.

$CD28^+$ T cells were cultured in quadruplicate samples in flat-bottomed 96-well microtiter plates in RPMI media containing 5% heat-inactivated fetal calf serum. Equal aliquots of cells were cultured for 18 hours and then pulsed for 6 hours with 1 uCi/well of

^{3}H-uridine, or for 72 hours and then pulsed for 6 hours with 1 uCi/well of ^{3}H-thymidine. The means and standard deviations (in cpm) were determined by liquid scintillation counting after cells were collected on glass fiber filters.

All cultures containing cells immobilized to plastic by anti-CD3 monoclonal antibodies were visually inspected to ensure complete cell harvesting. The failure of cells in these cultures to proliferate in response to PHA is the result of rigorous depletion of accessory cells, in vivo activated T cells, B cells, and CD11$^+$ (CD28$^-$) T cells by negative immunoabsorption as described in Specific Example II above. In each experiment, cells were stained with flourescein-conjugated anti-CD2 mAb OKT11 and flourescein-conjugated anti-CD28 mAb 9.3 and were shown to be over 99% and over 98% surface positive, respectively.

A representative experiment is illustrated in Figures 1 and 2. As shown in Figures 1 and 2, anti-CD28 by itself had no significant effect on uridine or thymidine incorporation, nor did it serve to augment proliferation induced either by immobilized anti-CD3 mAb G19-4 or chemically-induced T cell proliferation involving phorbol myristate acetate (PMA) and ionomycin (Iono). However, as shown in Figure 2, anti-CD28 did significantly increase the uridine incorporation of both sets of cells. In contrast, other monoclonal antibodies including anti-CD2 mAb OKT11 and anti-CD45 mAb 9.4 had no significant effect on uridine incorporation of anti-CD3 stimulated cells. This was not due to lack of effect of these antibodies on the cells, since both anti-CD2 and anti-CD7 monoclonal antibodies significantly augmented the proliferation of anti-CD3 stimulated cells. In separate experiments, the binding of isotype-matched mAbs to other T cell surface antigens (CD4, CD6, CD7 or CD8) and failed to mimic the effects observed with anti-CD28.

These data serve to confirm that the stimulation of activated T cells by CD28 has a unique phenotype which appears to directly enhance the rate of incorporation of a radioactive marker into the steady state RNA of T cells without directly enhancing T cell proliferation.

SPECIFIC EXAMPLE V

Increased Cellular Production of Human T_H1 Lymphokines by CD28 Stimulation Ex Vivo

Based on evidence from the in vitro systems it appeared that CD28 did not have a significant effect on cellular production of lymphokines unless they had undergone prior antigen activation or its equivalent. However, CD28 binding by the 9.3 mAb significantly enhanced the ability of anti-TCR/CD3 activated T cells to sustain production of human T_H1 type lymphokines. To test this effect in a physiologic setting, the activation of T lymphocytes in an ex vivo whole blood model was studied.

50-100 ml of venous blood was obtained by standard aseptic procedures from normal volunteers after obtaining informed consent. The blood was heparinized with 25 U/ml of preservative-free heparin (Spectrum, Gardenia, CA) to prevent clotting. Individual 10 ml aliquots were then placed on a rocking platform in a 15 ml polypropylene tube to maintain flow and aeration of the sample.

To assay for the effectiveness of CD28 stimulation on the induction of lymphokine gene expression, the production of TNF-α molecule was chosen as a model because of the extremely short half-life (approximately 15 minutes) of the protein in whole blood. 10 ml of whole blood isolated as described above was incubated with soluble anti-CD3 mAb G19-4 at a concentration of 1 μg/ml or anti-CD28 mAb 9.3 at a concentration of 1 μg/ml or a combination of the two antibodies. The plasma was assayed for TNF-α as described in Specific Example III at one and four hours. An example of one such experiment is shown in Table 1, which illustrates the significant increase in production of TNF-α by maximal stimulation of CD3 and co-stimulation of CD28.

TABLE 2

STIMULUS	TNF-α (pg/ml) 0hr	1hr	4hr
anti-CD3	4.5[a]	65.0	2.1
anti-CD28	4.5[a]	1.6	3.3
anti-CD3+ anti-CD28	4.5[a]	35.0	75.0

[a]value determined prior to addition of monoclonal antibody to aliquots of the venous sample

SPECIFIC EXAMPLE VI

Resistance of CD28-Induced T Cell Proliferation to Cyclosporine

The protocol used and results described herein are described in detail in June, C.H., et al., Mol. Cell. Biol., 7: 4472-4481 (1987), herein incorporated by reference.

T cells, enriched by nylon wool filtration as described by Julius, et al., Euro. J. Immunol., 3:645-649 (1973), were cultured at approximately 5 x 10^4/well in the presence of stimulators in the following combinations: anti-CD28 mAb 9.3 (100ng/ml) and PMA 1(ng/ml); or immobilized anti-CD3 mAb G19-4 (200ng/well); or PMA (100ng/ml). The above combinations also included fourfold titrations (from 25ng/ml to 1.6 μg/ml) of cyclosporine (CSP) (Sandoz, Hanover, NJ) dissolved in ethanol-Tween 80 as described by Wiesinger, et al., Immunobiology, 156:454-463 (1979).

^{3}H-thymidine incorporation was measured on day 3 of culture and the results representative of eight independent experiments are depicted in Figure 3. The arithmetic mean ± 1 standard deviation is depicted where the bar exceeds the size of the symbol. Proliferation of cells cultured in medium alone was 185 ± 40 cpm. The cyclosporine diluent alone did not affect cellular proliferation (data not shown). As shown in Figure 3, CD28-induced T cell

Table 3 below illustrates the effects of cyclosporine on CD3-induced proliferation of $CD28^+$ T cells cultured at approximately 5×10^4 cells/well in flat-bottomed 96-well microtites plates (CoStar, Cambridge, MA) under the following conditions: immobilized mAb G19-4; or immobilized mAb G19-4 and mAb 9.3 100ng/ml; or immobilized mAb G19-4 and PMA 1ng/ml; or mAb 9.3 100ng/ml and PMA 1ng/ml. Cyclosporine was prepared as above and included in the cultures at 0, 0.2, 0.4, 0.8, 1.2 μg/ml. ^{3}H-thymidine incorporation was determined on day 3 of culture as above. The percent inhibition of proliferation was calculated between $CD28^+$ T cells cultured in medium only or in cyclosporine at cyclosporine at 1.2 μg/ml. $CD28^+$ T cells cultured in the absence of cyclosporine were given cyclosporine diluent. ^{3}H-thymidine incorporation of cells cultured in medium, or PMA, or monoclonal antibody 9.3 only was less than 150 cpm. As shown in Table 3, co-stimulation of CD3 and CD28 resulted in a marked increase in the resistance of T cell proliferation to cyclosporine and the stimulation of CD28 in the presence of PMA resulted in a complete absence of cyclosporine suppression of T cell proliferation. Stimulation of CD28 together with immobilized anti-CD3 also resulted in resistance to suppression of T cell proliferation by the immunosuppressant dexamethasone.

TABLE 3

Effects of CD28 Stimulation on Cyclosporine Resistance on T Cell Proliferation

Mean [^{3}H]thymidine incorporation (kcpm) ± 1 SD at cyclosporine conc (μg/ml):

Stimulus	0	0.2	0.4	0.8	1.2	% Inhibition
CD3 mAb G19-4	77 ± 26	61 ± 6.8	52 ± 4.4	10 ± 3.4	8.2 ± 1.2	90
CD3 + CD28 mAb 9.3	123 ± 18	86 ± 2.3	63 ± 4.4	44 ± 6.4	43 ± 5.2	65
CD3 + PMA	145 ± 12	132 ± 2.8	123 ± 6.4	55 ± 3.6	56 ± 6.4	62
CD28 mAb 9.3 + PMA	111 ± 12	97 ± 5.6	107 ± 12	99 ± 14	112 ± 2.4	<0

SPECIFIC EXAMPLE VII

Human T_H1 Lymphokine Secretion in the Presence of Cyclosporine

As described in Specific Example III, $CD28^+$ T cells were cultured in the presence of various stimulators. Culture supernatants were harvested at 24 hours and serial dilutions assayed for IL-2, TNF-α/LT, IFN-γ, and GM-CSF as previously described. Separate aliquots of cells were recovered 48 hours after stimulation and assayed for the percentage of cells in late stages of the cell cycle ($S+G_2+M$).

When cyclosporine at 0.6 μg/ml was included in the test protocol, as shown in Table 4 (which also incorporates the data of Specific Example III for comparison), $CD28^+$ T cells were found to secrete the human T_H1 lymphokines in the presence of cyclosporine in cultures stimulated with mAb 9.3 and PMA; or immobilized mAb G19-4 and mAb 9.3; or PMA and ionomycin and mAb 9.3. Human T_H1 lymphokine production induced by immobilized mAb G19-4; or by PMA with ionomycin was, however, completely suppressed in the presence of cyclosporine.

TABLE 4

Increased Cellular Production of Human T_H1 Lymphokines by TNF-U/LT,

STIMULUS	IL-2 (U/ml)	TNF-α/LT (U/ml)	IFN-γ (U/ml)	GM-CSF (U/ml)	$S+G_2+M$ (%)
Medium	<2	0	0	0	4.6
PMA	<2	0	0	NT	5.5
Anti-CD28	<2	5	0	0	6.5
Anti-CD28+PMA	435	300	24	150	48.9
Anti-CD28+PMA +CSP	192	200	12	NT	49.3
Anti-CD3'	36	50	24	120	39.7
Anti-CD3'+CSP	<2	0	0	NT	14.5
Anti-CD3'+Anti -CD28	1200	400	74	1050	44.7
Anti-CD3'+Anti-CD28 +CSP	154	200	9	NT	48.6
Ionomycin	<2	0	0	NT	6.6
Ionomycin+PMA	200	5	37	NT	43.6
Ionomycin+PMA +CSP	<2	0	0	NT	8.1
Ionomycin+PMA+Anti- CD28	1640	320	128	NT	43.5
Ionomycin+PMA+Anti- CD28+CSP	232	120	15	NT	47.6

' = immobilized
NT = not tested

SPECIFIC EXAMPLE VIII

Human T_H1 Lymphokine mRNA Expression in the Presence of Cyclosporine

In order to further examine whether CD28 stimulation led to cyclosporine-resistant human T_H1 lymphokine gene expression as well as secretion, the ability of cyclosporine to suppress induction of IL-2, TNF-α, LT, IFN-γ, and GM-CSF following stimulation by various stimulators was tested. Specifically, $CD28^+$ T cells were cultured at 2×10^6/ml in complete RPMI medium (GIBCO, Grand Island, NY) with 5% FCS (MED). Individual aliquots of $CD28^+$ T cells were incubated for 6 hours in the presence or absence of 1.0 μg/ml cyclosporine with PMA 3ng/ml and anti-CD28 mAb 9.3 (1mg/ml); or with immobilized anti-CD3 mAb G19-4 (1 μg/well); or with immobilized mAb G19-4 (1 μg/well) and mAb 9.3 (1ng/ml). $CD28^+$ T cells were harvested, total cellular RNA isolated and equalized for ribosomal RNA as previously described by Thompson, et al., Nature, 314:363-366 (1985).

Northern blots were prepared and hybridized sequentially with ^{32}P-labeled, nick-translated gene specific probes as described by June, C.H., et al., Mol. Cell. Biol., 7:4472-4481 (1987). The IL-2 probe was a 1.0 kb Pst I cDNA fragment as described by June, C.H., et al., Mol. Cell. Biol., 7:4472-4481 (1987); the IFN-γ probe was a 1.0 kb Pst I cDNA fragment as described by Young, et al., J. Immunol., 136:4700-4703 (1986). The GM-CSF probe was a 700 base pair EcoR I-Hind III cDNA fragment as described by Wong, et al., Science, 228:810-815 (1985); the 4F2 probe was a 1.85 kb EcoR I cDNA fragment as described by Lindsten, et al., Mol. Cell. Biol., 8:3820-3826 (1988); the IL4 probe was a 0.9 kb Xho I cDNA fragment as described by Yokota, et al., PNAS (USA), 83:5894-5898 (1986); and the human leukocyte antigen (HLA) probe was a 1.4 kb Pst I fragment from the HLA-B7 gene as described by Lindsten, et al., Mol. Cell. Biol., 8:3820-3826 (1988). TNF-α and LT specific probes were synthesized as gene specific 30 nucleotide oligomers as described by Steffen, et al., J. Immunol., 140:2621-2624 (1988) and Wang, et al., Science, 228:149-154 (1985). Following hybridization, blots were washed and exposed to autoradiography at -70°C. Quantitation of band densities was performed by densitometry as described in Lindsten, et al., Mol. Cell. Biol., 8:3820-3826 (1988).

As shown by the Northern blot of Figure 4, stimulation by

mAb 9.3 with PMA and by mAb 9.3 with mAb G19-4 led to human T_H1 lymphokine gene expression that exhibited resistance to cyclosporine. In contrast, stimulation by mAb G19-4 alone was completely suppressed in the presence of cyclosporine.

SPECIFIC EXAMPLE IX

In Vivo Activation of T Cells by CD28 Stimulation

$F(ab')_2$ fragments of mAb 9.3 were prepared as described by Ledbetter, J. A., et al., J. Immunol., 135:2331-2336 (1985). Purified and endotoxin-free $F(ab')_2$ fragments were injected intravenously at 1 mg/kg of body weight over a 30 minute period into a healthy macaque (M. nemestrina) monkey. On days 2 and 7 after injection, 5 ml of blood was drawn and tested.

Peripheral blood lymphocytes from the monkey's blood were isolated by density grandient centrifugation as described in Specific Example II. Proliferation of peripheral blood mononuclear cells in response to PMA (1ng/ml) was tested in the treated monkey and a control animal (no $F(ab')_2$ fragment treatment) in triplicate as described in Specific Example IV. Proliferation was measured by the uptake of ^3H-thymidine during the last 6 hours of a three-day experiment and the results shown in Figure 5. Means of triplicate culture are shown, and standard errors of the mean were less than 20% at each point. As shown in Figure 5, stimulation of CD28 by the $F(ab')_2$ mAb 9.3 fragment increased T cell proliferation in vivo.

It should be appreciated that a latitude of modification, change or substitution is intended in the foregoing disclosure and, accordingly, it is appropriate that the appended claims be construed broadly and in a manner consistent with the spirit and scope of the invention herein.

What Is Claimed Is:

1. A method of immunotherapy comprising the step of:

selectively regulating the in vivo level of a human T-cell lymphokine by administering a therapeutically effective amount of a ligand to a patient having a population of activated T cells, said ligand having binding specificity for at least a portion of the extracellular domain of CD28.

2. The method of Claim 1, wherein said step of regulating further comprises the step of selecting a ligand which has a stimulatory effect on the CD28 pathway.

3. The method of Claim 1, wherein said step of regulating further comprises the step of selecting a ligand which has an inhibitory effect on the CD28 pathway.

4. The method of Claim 1, wherein said T-cell lymphokine is a lymphokine selected from the group consisting of IL-2, TNF-α, LT, IFN-γ and GM-CSF.

5. The method of Claim 1, wherein said ligand comprises at least a portion of an anti-CD28 antibody.

6. The method of Claim 5, further comprising the step of isolating said anti-CD28 antibody.

7. The method of Claim 5, wherein said anti-CD28 antibody is an antibody having the characteristic of inducing the proliferation of cyclosporine treated T cells in vitro when used in conjunction with PMA.

8. The method of Claim 5, wherein said ligand comprises the $F(ab')_2$ fragment of monoclonal antibody 9.3.

9. The method of Claim 5, wherein said ligand comprises a monoclonal antibody having the CD28 binding characteristics of monoclonal antibody 9.3.

10. The method of Claim 5, wherein said ligand comprises a monoclonal antibody having the CD28 binding characteristics of Kolt-2.

11. The method of Claim 5 wherein said ligand is a chimaeric antibody.

12. A method of immunotherapy for selectively enhancing a T cell-mediated immune response specific for an antigen to which the recipient of said immunotherapy is sensitized by in vivo exposure thereto, said recipient thereby having a population of T cells undergoing activation, said method comprising the steps of:

a) selecting a CD28 stimulator capable of binding to the extracellular domain of the CD28 molecule;

b) providing said stimulator in a biologically compatible form suitable for administration in vivo; and

c) administering said stimulator in said biologically compatible form in an amount sufficient for and for a time sufficient for said stimulator to bind to at least a portion of said population of T cells undergoing activation.

13. The method of Claim 12, wherein said T cells are undergoing activation by the binding of said antigen to the TCR/CD3 complex.

14. The method of Claim 12, wherein said CD28 stimulator comprises at least a fragment of an anti-CD28 antibody, said antibody having the characteristic of inducing the proliferation of cyclosporine-treated T cells when used in conjunction with PMA in vitro.

15. The method of Claim 12, wherein said antigen is produced by a tumor cell.

16. The method of Claim 12, wherein said antigen is produced by an infected cell.

17. The method of Claim 10, wherein the CD28 stimulation is at least a portion of an anti-CD28 chimaeric antibody.

18. The method of Claim 14, wherein said antibody is monoclonal antibody 9.3.

19. The method of Claim 14, wherein said antibody has the CD28 binding characteristics of Kolt-2.

20. The method Claim 18, wherein said fragment is the $F(ab')_2$ fragment.

21. A method of augmenting a T-cell mediated immune response in an immunosuppressed patient comprising the steps of:

a) providing an anti-CD28 antibody, said antibody having the characteristic of inducing the in vitro proliferation of cyclosporine-treated T cells when said antibody is used in conjunction with PMA;

b) providing at least a portion of said anti-CD28 antibody in a biologically compatible form suitable for administration in vivo; and

c) administering said portion of said anti-CD28 antibody in said biologically compatible form to said immunodepressed patient in a therapeutically effective amount, said amount being sufficient to enhance a T cell-mediated immune response.

22. The method of Claim 21, wherein said portion of said anti-CD28 antibody binds to the extracellular domain of CD28.

23. The method of Claim 22, wherein said antibody comprises the $F(ab')_2$ fragment of monoclonal antibody 9.3.

24. The method of Claim 22, wherein said antibody comprises a monoclonal antibody having the CD28 binding characteristics of Kolt-2.

25. The method of Claim 22, wherein said antibody is a chimaeric antibody.

26. A method for substantially increasing the cellular production of selected T cell lymphokines by a population of human T cells comprising the steps of:

a) providing an in vivo population of T cells undergoing activation, wherein said T cells are activated by the binding of a first ligand to a stimulatory site of the surface of said T cell to stimulate said site, wherein said stimulation of at least a portion of said population is maximized; and

b) stimulating the CD28 T cell surface molecule by binding said molecule with a second ligand having binding specificity for the extracellular domain of said CD28 molecule.

27. The method of Claim 26, wherein said first ligand is an antigen.

28. The method of Claim 27, wherein said selected T cell lymphokines are lymphokines selected from the group consisting of IL-2, TNF-α, LT, IFN-γ and GM-CSF.

29. The method of Claim 27, wherein said second ligand is at least a fragment of an antibody.

30. The method of Claim 29, wherein the antibody is a chimaeric antibody.

31. The method of Claim 29, wherein said antibody is a monoclonal antibody.

32. The method of Claim 31, wherein said monoclonal antibody is mAb 9.3.

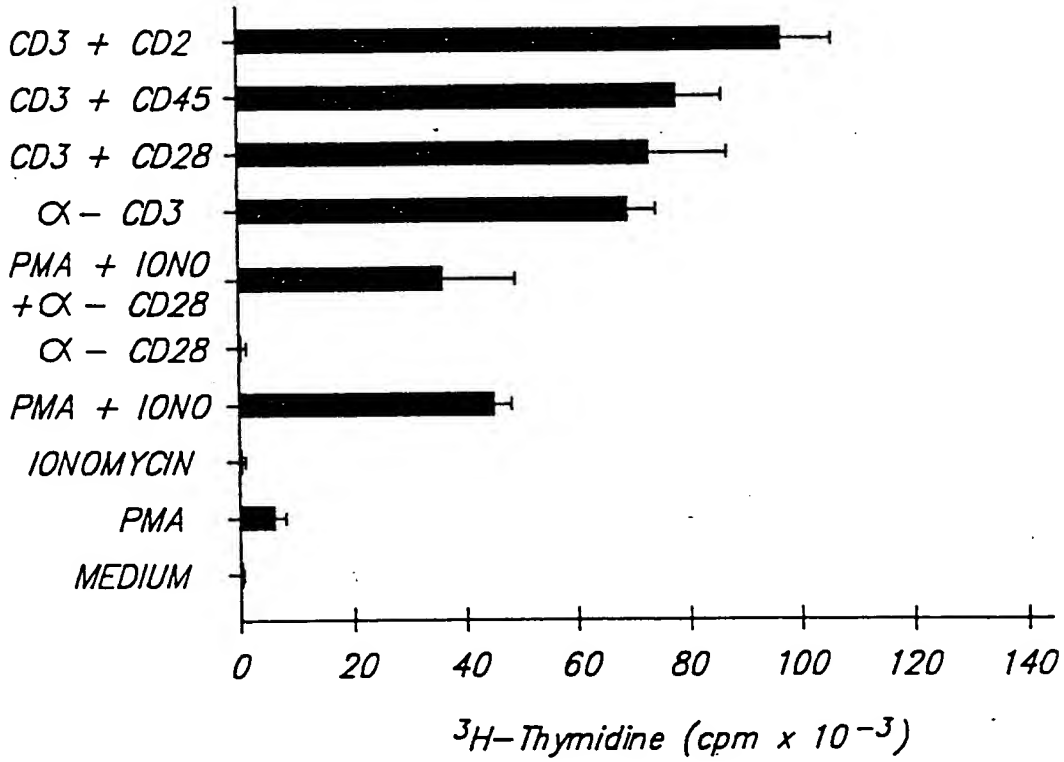
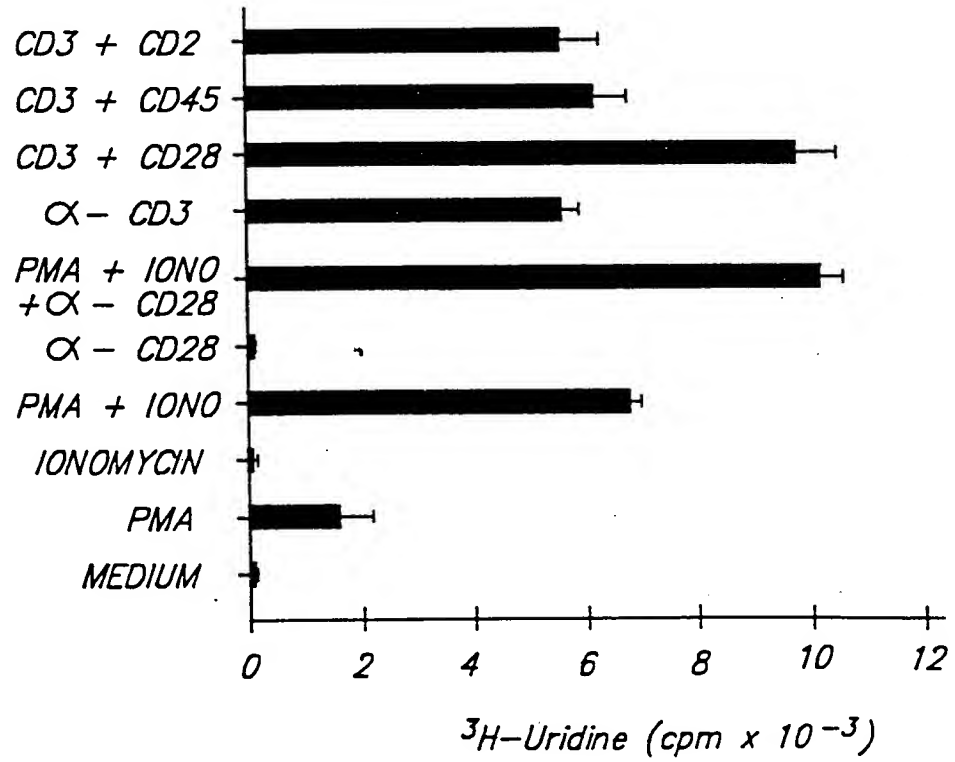

CD3 + CD2
CD3 + CD45
CD3 + CD28
∝ – CD3
PMA + IONO +∝ – CD28
∝ – CD28
PMA + IONO
IONOMYCIN
PMA
MEDIUM
0
20
40
60
80
100
120
140
^{3}H–Thymidine (cpm x 10^{-3})

Fig. 1.


CD3 + CD2
CD3 + CD45
CD3 + CD28
∝ – CD3
PMA + IONO +∝ – CD28
∝ – CD28
PMA + IONO
IONOMYCIN
PMA
MEDIUM
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2
4
6
8
10
12
^{3}H–Uridine (cpm x 10^{-3})

Fig. 2.

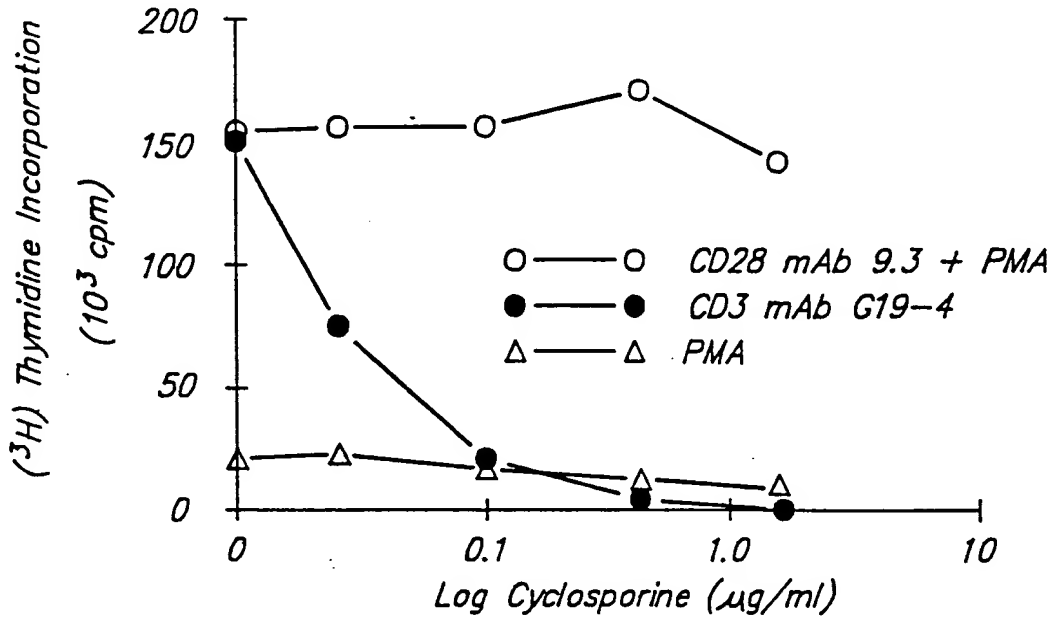

200
150
100
50
0
0
0.1
1.0
10
(3H) Thymidine Incorporation
(10³ cpm)
Log Cyclosporine (µg/ml)
CD28 mAb 9.3 + PMA
CD3 mAb G19-4
PMA

Fig. 3.

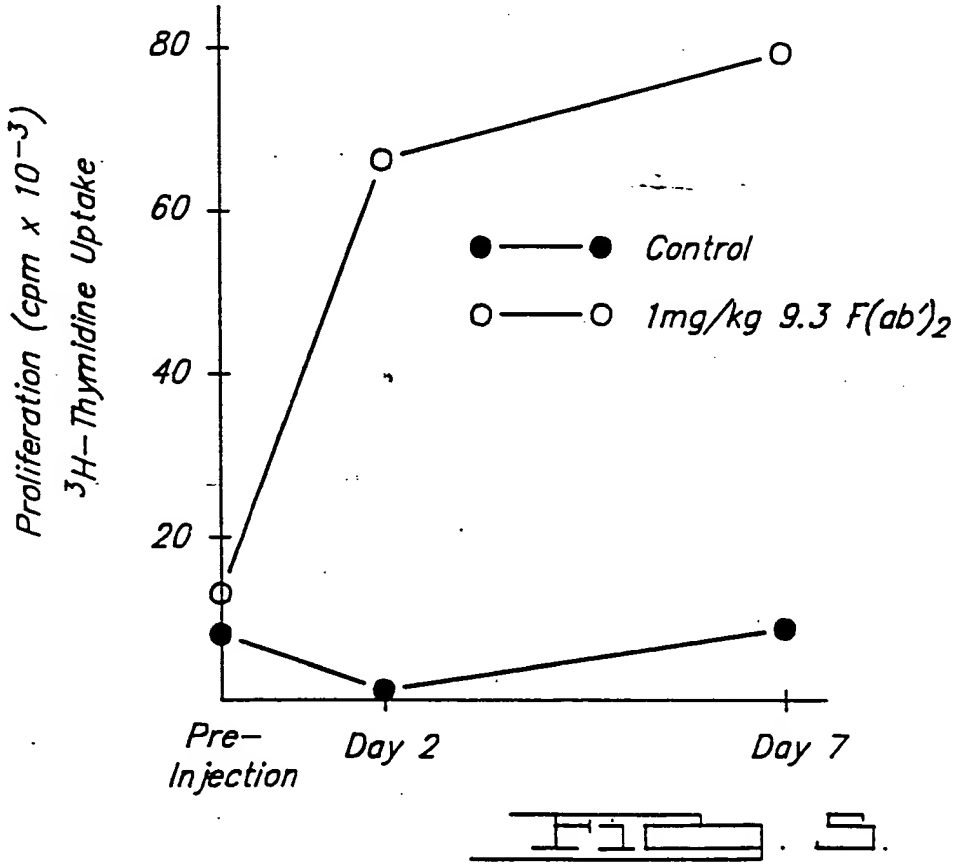
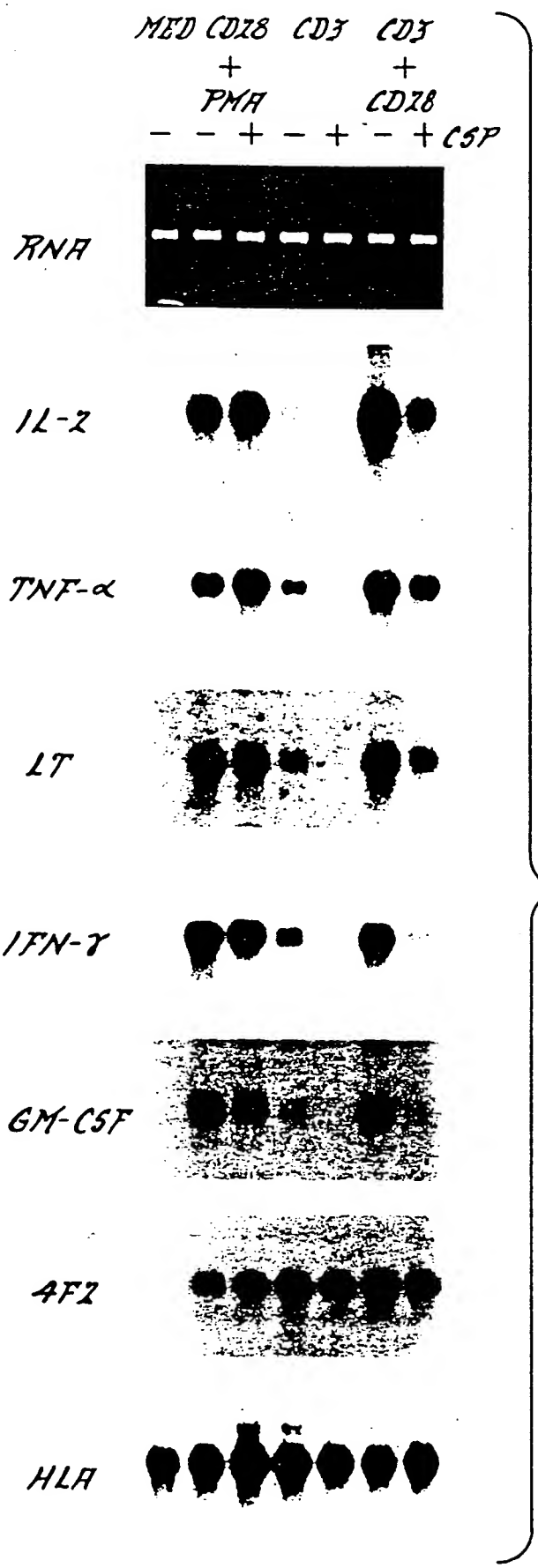

80
60
40
20
Proliferation (cpm x 10⁻³)
³H-Thymidine Uptake
Control
1mg/kg 9.3 F(ab')2
Pre-
Injection
Day 2
Day 7

Fig. 5.



TH1 Lymphokine mRNA Expression in the Presence of Cyclosporine

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05304

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/395

U.S. CL.: 424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/85.8; 530/387,388; 436/548

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

ONLINE COMPUTER SEARCH OF CHEMICAL ABSTRACTS AND BIOSIS 1967-1990
SEARCH TERMS: CD 28 T CELL SURFACE PROTEIN, ANTIBODIES THERE TO

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	U.S., A, 4,654,210 (KUNG) 31 March 1987. See entire document.	1-32
Y	J. of Immunology, Volume 136, Number 9, issued May 1983 (U.S.A) MARTIN, "A 44 Kilodalton Cell Surface Homodimer Regulates Interleukin 2 Production By Activated Human T. Lymphocytes", see pages 3282-3286.	1-32
Y	Molecular and Cellular Biology, Volume 7, Number 12, issued December 1987 (U.S.A.) JUNE, "T-Cell Proliferation Involving the CD 28 Pathway Is Associated with Cyclosporine-Resistant Interleukin 2 Gene Expression", see pages 4472-4481.	1-32

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

14 February 1990

Date of Mailing of this International Search Report

28 FEB 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

BLONDEL HAZEL, PRIMARY EXAMINER

Form PCT/ISA/210 (second sheet) (Rev.11-87)